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REVIEW

Regulation of C-reactive protein conformation in inflammation

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Abstract
C-reactive protein (CRP) is a non-specific diagnostic marker of inflammation and an evolutionarily conserved protein with roles in innate immune signaling. Natural CRP is composed of five identical globular subunits that form a pentamer, but the role of pentameric CRP (pCRP) during inflammatory pathogenesis remains controversial. Emerging evidence suggests that pCRP can be dissociated into monomeric CRP (mCRP) that has major roles in host defenses and inflammation. Here, we discuss our current knowledge of the dissociation mechanisms of pCRP and summarize the stepwise conformational transition model to mCRP to elucidate how CRP dissociation contributes to proinflammatory activity. These discussions will evoke new understanding of this ancient protein.

Keywords C-reactive protein · Pentameric C-reactive protein · Monomeric C-reactive protein · Conformation · Dissociation · Inflammation

Introduction

C-reactive protein (CRP) is an acute phase protein primarily expressed and secreted by the liver. In response to tissue injury or infection, the plasma concentrations of CRP can increase rapidly from baseline levels of less than 1 μg/mL within 48 h. In addition, the CRP concentration also increases in chronic inflammatory diseases, including cardiovascular and autoimmune disease. Due to the correlation between CRP and inflammation, CRP has attracted wide attention as a non-specific marker to evaluate and monitor the development of infection and inflammation, and as a prognostic marker for cardiovascular events. However, emerging evidence indicates that CRP not only indicates inflammation, but also regulates innate immunity and inflammatory progression.

CRP exists in both native pentameric (pCRP) and monomeric conformations (mCRP). Although this has long been recognized, the relationship between different CRP conformations and their respective roles in inflammation is less well understood. Contradictory conclusions on the physiological functions of CRP have resulted. Over the past decade, our understanding of the mechanism by which pCRP depolymerizes to mCRP, and the differing roles of these two conformational CRP in the development of inflammation has rapidly increased. These progressions not only explained the reason of confusing findings, but also motivated people to revive interest in the ancient protein and to reassess its contribution to many diseases. This article will review the latest advances in our understanding of the CRP conformational changes and their involvement in the development of inflammation.

Structural characteristics of CRP provide insight into conformational changes

Native CRP is pentameric

Native CRP secreted by the liver consists of five identical subunits that are 206 amino acids in length. The crystal structure of CRP shows that five subunits with the same orientation tightly assemble via non-covalent bonds to form a discoid pentamer with a central void [1]. Each CRP subunit contains a hydrophobic core composed of two antiparallel beta-sheets stabilized by the only intrachain disulfide

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bond. On the opposite side of the disulfide bond, the subunit can bind two calcium ions (Fig. 1) [1] which are important for structural integrity and pentamer stability. When pCRP is stored in buffers lacking calcium or containing EDTA, pCRP depolymerizes [2, 3] leading to loosely associated subunits that can be hydrolyzed by proteases [4]. In addition, the calcium-dependent binding to ligands, e.g., phosphocholine (PC), highlights the importance of calcium ions for CRP functionality. From the structure of CRP co-crystallized with PC and calcium, the major interaction occurs between the phosphate group of PC and the calcium bound to CRP (Fig. 1) [4]. In addition to PC, pCRP can bind diverse ligands including polysaccharides, polycations protein and chromatin, through its recognition face [5]. Ligand binding leads to subunit rotation facilitating the interaction of CRP with complement C1q or FcγR, FcαR, SR-A, and LOX-1 receptors, allowing it to participate in host defense and immune regulation [5].

mCRP is a naturally occurring isoform of CRP

pCRP incubated with 8 mol/L urea containing 10 mmol/L EDTA or heated at 63 °C for 5 min can be dissociated into its subunit, termed mCRP [2, 6]. The dissociation to mCRP is accompanied by a loss of secondary structural elements and significant alterations in tertiary structure contributing to mCRP epitope expression [7–11]. Immunohistochemical analyses demonstrated that CRP located in inflammatory tissue can be recognized by antibodies recognizing residues 199–206 [12–15]. The epitope exists in subunit of pCRP and is exposed when pCRP undergoes conformational changes. Additionally, autoantibodies against mCRP were found in active lupus nephritis [16], indicating that mCRP antigens are naturally expressed in vivo. Crawford et al. [17] reported that mCRP-microparticles can be quantified in individuals with peripheral artery disease and that the measured levels do not correlate with pCRP levels. Zhang et al. [18] successfully quantified human serum mCRP using ELISA techniques based on commercially available reagents and demonstrated that mCRP is a more reliable marker than pCRP in several skin-related autoimmune disorders. The culmination of these studies highlights that mCRP is a naturally occurring isoform of CRP that plays a significant role in inflammatory processes.

pCRP* is the predominant conformation of CRP deposited in inflammatory tissue

Even though CRP deposited in inflamed tissue can be recognized by antibodies–recognizing residues 199–206, this cannot reflect quaternary structure of deposited CRP. Braig et al. [11] reported that pCRP bound to microvesicles originated from LPS–stimulated cells could undergo structural changes that lead to the formation of another pCRP isoform. The isoform, termed as pCRP*, expresses subunit epitope but maintains pentameric symmetry. pCRP* should resemble the intermediate conformation emerging in the process of pCRP dissociation. Braig et al. [11] further demonstrated that pCRP* is the predominant conformation of CRP deposited in inflamed or injured tissue. Similar to mCRP, pCRP*

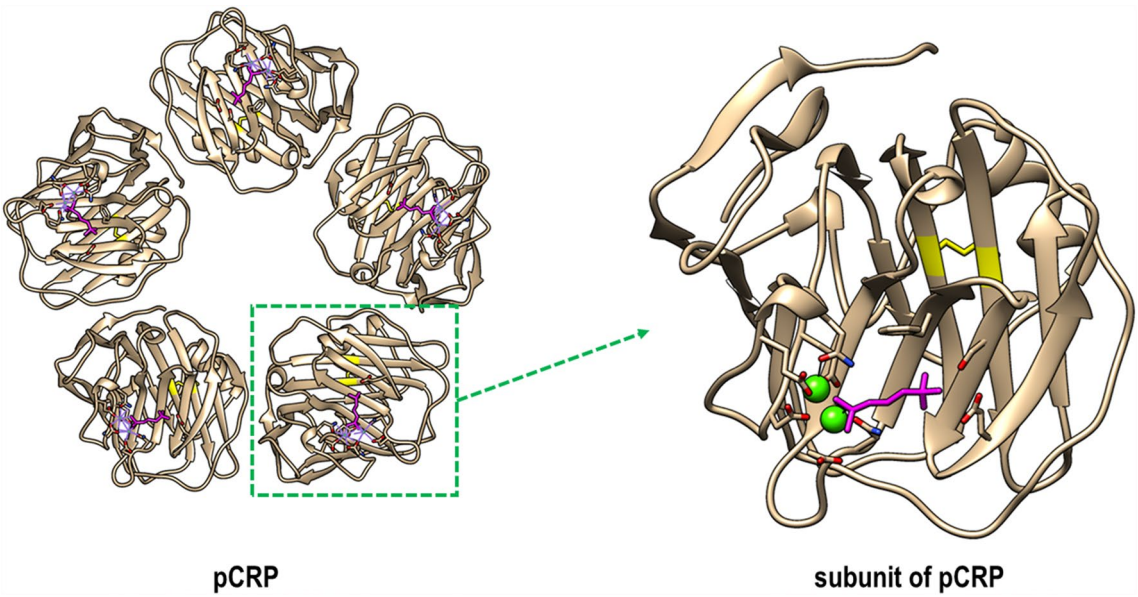

Fig. 1 Structure of pCRP (PDB:1B09). pCRP consists of five identical subunits. Each subunit contains a hydrophobic core stabilized by the only intrachain disulfide bond (yellow). On the opposite side of the disulfide bond, the subunit can bind two calcium ions (green), which participate in the binding of PC (purple)

can exert serious proinflammatory activities including activation of complement, expression of cell adhesion molecules on endothelial cells and recruitment of leukocytes.

Both pCRP* and mCRP are proinflammatory isoforms of CRP. However, the activity of pCRP* is also different from that of mCRP. pCRP* has stronger ability to bind C1q and activate the classical complement pathway than mCRP [11]. By contrast, mCRP not only activates the complement cascade but also negatively regulates the degree of inflammatory response by aiding opsonization. Mihlan et al. [19] demonstrated that mCRP can recruit and bind Factor H to enhance C3b inactivation and inhibit the expression of proinflammatory cytokines. O'Flynn [20] showed that mCRP is an inhibitor of properdin and can limit aggravation of tissue injury by inhibiting complement activation of properdin-directed alternative pathway. Additionally, mCRP can efficiently promote uptake of mCRP-decorated vesicles by macrophages due to its more stretched and disordered structure [11]. Thus pCRP* and mCRP may represent different time points in cascade of inflammation.

Membrane–induced dissociation of pCRP releases its proinflammatory activity

In vitro studies revealed that the incubation of human coronary artery endothelial cells (HCAEC) with mCRP for 4 h increased the secretion of interleukin-8 (IL-8), and the expression of monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin and vascular adhesion molecule-1 (VCAM-1) [21]. However, proinflammatory effects were observed only after incubation with pCRP for 24 h [21–23]. In addition, Ji et al. [8] demonstrated that mCRP could strongly bind to C1q and activate the classical complement pathway after immobilization on microtiter plates. By contrast, pCRP exhibited low affinity to C1q [8]. Although pCRP also bind C1q after immobilization, obvious conformation alteration occurred in pCRP result in exposure of mCRP epitope [8]. These evidences indicated that conformational changing of pCRP releases its proinflammatory activities.

Ji et al. [7] elucidated the process of pCRP structural alterations that lead to the formation of mCRP under physiological condition. The results demonstrated that binding of pCRP to EggPC/lysoPC–fused liposomes or apoptotic cell membrane domains containing lysoPC rapidly induces steric structural changes leading to the decreased tryptophane fluorescence and exposure of neoepitopes in the pCRP subunit. This reflect the changing of tertiary and oligomeric structure of pCRP. Results from electron microscopy studies show that pCRP bound to monolayers containing lypoPC primarily exhibit pentameric structure for 30 min [7]. After 2 h, pCRP loses its cyclic structure evidently [7]. If continued to incubate for 24–48 h, pCRP completely disassociates into subunits [7].

In the past few years, the mechanism by which CRP interacts with cell membranes has been studied intensively. Treating apoptotic cells with exogenous PLA_2 to catalyze lysoPC generation can significantly promote pCRP binding and the expression of epitopes in CRP subunits [7]. In vivo studies supported these findings by demonstrating that blocking PLA_2 could abrogate pCRP dissociation, blunting the proinflammatory activity of CRP [24]. This indicates that membrane domains containing lysoPC are the major location for pCRP binding on cell membranes. In addition to lysoPC, lipid rafts, membrane domain containing high content of cholesterol and sphingomyelin, also participate in pCRP binding and dissociation [7]. On one hand, cholesterol and PC headgroup of sphingomyelin are natural ligand of pCRP. On the other hand, the assembly pattern of cholesterol and sphingomyelin in lipid rafts may be analogous to environment of altered membrane [7]. Dissociation also occurs after pCRP attaches to plastic surfaces [25], immobilized PC and polylysine [7]. These results suggest that the hydrophobic microenvironment of cell surface and multipoint attachment are necessary for the binding and dissociation of pCRP [7]. Membrane curvature is an important factor influencing this process [26]. This may explain why pCRP* formation can occur on microvesicles rather than apoptotic bodies which have diameter of 1–5 μm and are larger than microvesicles [11, 27]. In addition, membrane fluidity or lateral movement of ligand also promotes the separation of dissociated CRP subunits on the cell surface [7].

The mechanism of membrane-induced pCRP dissociation may reflect the regulation of CRP structure and function under pathological conditions. Eisenhardt et al. [13] demonstrated that activated platelets mediate the dissociation of pCRP to mCRP by exposing lysoPC on their cell membranes, thereby unmasking the proinflammatory effects of CRP and limiting its effects on atherosclerotic plaques. Habersberger et al. [28] reported that circulating microparticles (MP) enriched in lysoPC were more abundant following myocardial infarction. These MPs not only convert pCRP into mCRP, but also carry and transport mCRP to the surface of endothelial cells exerting proinflammatory functions. Strang et al [29] demonstrated that mCRP can be detected in the cerebral cortex obtained from Alzheimer's disease (AD) patients, and co-localize with β-amyloid plaques. Further studies have demonstrated that aggregated β-amyloid plaques can dissociate pCRP to mCRP [29], which may reflect an important mechanism of CRP's involvement in AD. The culmination of these in vivo data not only promoted the development of membrane induced pCRP dissociation mechanisms, but also sparked further interest into the physiological roles of different conformational CRP.

mCRP exerts its proinflammatory activity primarily via interaction with lipid raft

FcγRIIIb (CD16) has been identified as an mCRP receptor. Through its interaction with CD16, mCRP can delay neutrophil apoptosis [30] and induce its activation [31], adherence, and migration [32]. mCRP also activates HAECs [33, 34] and stimulates monocytes to generate reactive oxygen species (ROS) [12] through its interaction with CD16. However, the activity of mCRP is only partially inhibited by CD16 blockade [30, 33] indicating that other mCRP—cell membrane interactions occur. Removal of surface protein and polysaccharide by trypsin or polysaccharide lyases had no effect on mCRP binding [9]. Further evaluation showed that washing with high salt, acid, or alkaline after binding of mCRP with HAECs cannot detach mCRP from cells [9]. These results indicate that the interaction of mCRP with cells is independent of peripheral receptor, and cell-bound mCRP may be incorporated into cell membrane as an integral membrane protein. Indeed, the way of the interaction between mCRP and cell membrane is consistent with the structural characteristics of mCRP. These characteristics, including a hydrophobic main structure and a prominent α-helix, reflect a possibility of mCRP as a membrane protein.

Lipid raft, enriched in cholesterol, sphingomyelin and other long-chain saturated fatty acids, is microdomain commonly found in cell membrane. Many important components of cell signaling networks, including GPI-connexin, G protein-coupled receptor (GPCR), epidermal growth factor receptor (EGFR) and T cell receptor (TCR), either reside in lipid rafts constitutively or are recruited into lipid raft rapidly in response to a specific stimulus. Ji et al. [9] showed that mCRP can be significantly incorporated into monolayers containing lipid raft components (lecithin/ cholesterol/ sphingolipid) by hydrophobic insertion, independent of Fcγ and proteoglycan receptors. Ji el al. [9] also showed that mCRP colocalized with cholera toxin β subunit, a marker of lipid rafts in U937 cells lacking CD16. Additionally, the use of MβCD and nystatin to destroy lipid rafts in HAECs can completely inhibit mCRP binding leading to reduced ROS production and reduced expression or secretion of ICAM-1, VCAM-1, E-selectin, MCP-1 and IL-8 [9].

Detailed studies have revealed that cholesterol is a key component mediating the insertion of mCRP into lipid rafts. The interaction between mCRP and liposomes or endothelial cells is dependent on the concentration of cholesterol in the membranes [9]. Sequence analysis found that the cholesterol binding sequence (CBS: aa 35–47) and C-terminal octapeptide (aa 199–206) in mCRP are directly involved in lipid raft association [9, 35]. Preincubation of mCRP with 8C10 mAb (recognizes aa 22–45) suppresses about 40% of mCRP insertion into monolayer containing raft components, whereas the suppression exceeds 60% when using 3H12 mAb (recognizes aa 199–206) [9]. The evidence demonstrated that C-terminal octapeptide (aa 199–206) is a major determinant of mCRP insertion into lipid raft. Structural analysis showed that in pCRP, CBS is buried in the hydrophobic core of the subunit, whilst the C-terminal octapeptide is located at the interface between subunits [35]. Following the dissociation of pCRP on membranes, the sequences become exposed. mCRP then binds to cholesterol through CBS and inserts into lipid rafts through its C-terminal octapeptide [9]. However, mCRP is sensitive to proteolysis after dissociated from pCRP [36, 37]. Some studies reported that mCRP can be digested by membrane protease of activated neutrophils to generate many bioactive peptides, including peptide 201–206 [38, 39], which could attenuate recruitment of neutrophils [39, 40], platelet activation [41], and platelet capture of neutrophils [41], thereby limiting the process of inflammation. These mechanisms explain how cell membranes regulate CRP structure and function and how mCRP interacts with membranes following its formation.

Reduction of disulfide bond enhances the proinflammatory activity of mCRP

mCRP when obtained from different preparation methods displays significantly altered biological activity. Although both mCRP prepared by denaturing pCRP with urea and by gene recombination express same epitope, the latter have stronger proinflammatory activity. Ji et al. [42] reported that recombinant mCRP binds more strongly to LDL. Wang et al. [10] reported that recombinant mCRP has stronger abilities to bind complement C1q and further activate complements, to activate HAECs cells to express proinflammatory cytokines (MCP-1, IL-8) and cell adhesion molecules (VCAM-1, ICAM-1, E-selectin), and to promote adhesion of monocytes to HAECs cells. However, following the reduction of intrachain disulfide bond, mCRP originating from urea denatured pCRP exhibits similar activity to recombinant mCRP. This indicates that intrachain disulfide bond is an important switch of mCRP function. This hypothesis was proven by Wang et al. who reported that mCRP frequently colocalized with thioredoxin in atherosclerotic plaques [10].

Each CRP subunit contains two evolutionarily conserved cysteine residues (Cys36, Cys97) that form intrachain disulfide bond to stabilize the hydrophobic core of the subunit. Following disulfide bond reduction, CRP subunits exist in an extended configuration [3]. However, disulfide bonds are only reduced when pCRP dissociates into monomeric subunits, indicating that pCRP

dissociation is a prerequisite for the reduction of disulfide bonds [10]. Based on the location of Cys36 in CBS, it is speculated that the reduction of disulfide bond or the mutation of cysteine residues improves CBS exposure. In fact, removing the disulfide bond by reduction or mutation can promote the interaction of mCRP with lipid rafts [10], contributing to the amplified proinflammatory activity of reduced mCRP.

Model of stepwise conformation transition of CRP to modulate inflammatory response

In summary, conformational changes that lead to proinflammatory CRP activity should have the following process (Fig. 2). Firstly, pCRP binds to multivalent ligands in the membranes of damaged or activated cells, which overcomes the stabilizing effects of Ca^{2+} that maintain pCRP. Subsequently, pCRP undergoes rapid and partial conformational

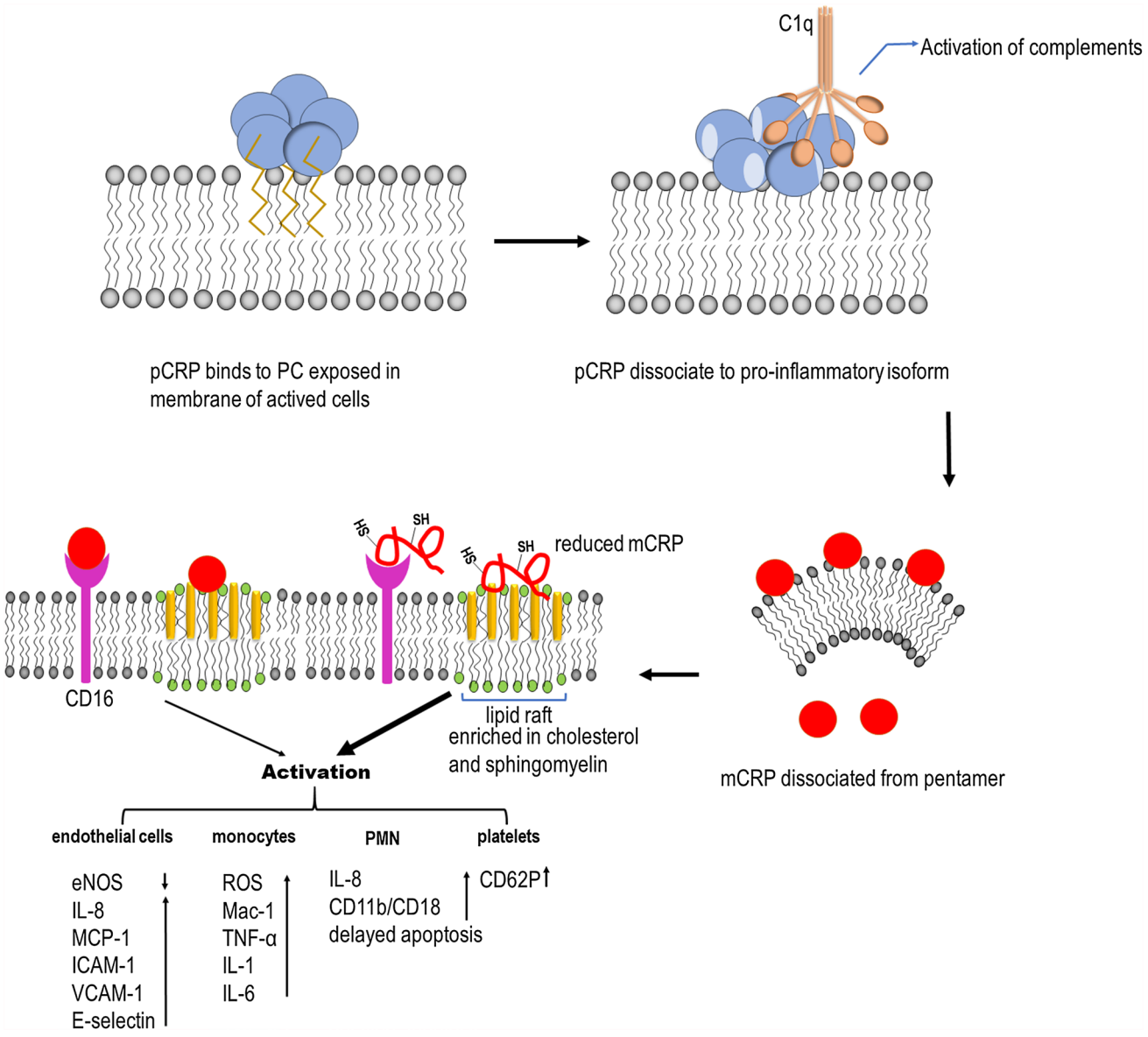

Fig. 2 Model of stepwise dissociation of pCRP to mCRP. pCRP binds to PC exposed in the membranes of activated cells and undergoes significant conformational changes leading to the exposure of subunit epitopes. At this point, dissociated CRP subunits are still restricted to a pentameric symmetry. The conformation has stronger ability to bind C1q facilitating activation of complements. Next, these subunits are detached from pentamer and form membrane-bound mCRP or free mCRP. mCRP can activate various of cells via interaction with CD16 and lipid raft leading to a series of inflammatory response. The proinflammatory activity of mCRP can be exaggerated following reduction of intrachain disulfide bond. The figure is cited from Caprio's work [43] and modified properly

changes which disrupt the steric hindrance from the neighboring subunit and lead to the exposure of subunit epitopes and C1q binding site. At this point, dissociated CRP subunits are still restricted to a pentameric symmetry (pCRP*). However, the altered conformation can exert serious of proinflammatory activities and activate the classical complement pathway by binding C1q. Thus, the rapid conformation alteration of pCRP to pCRP* may contribute to acute phase response, and complement mediated tissue injury and clearance of endogenous cells or infected bacteria. After a longer duration, CRP subunits separate from each other under the action of membrane fluidity to form mCRP, which is accompanied by a loss of secondary structural elements and significant alterations in tertiary structure. Since altered ligand interaction ability, mCRP exhibit distinct bioactivities. mCRP can effectively insert into cell membrane and promote cellular response by exposed CBS and C-terminal sequence. Additionally, mCRP can regulate LDL metabolism and complement activation. Reductive substances located on the damaged or activated cell membranes then reduce the disulfide bond in mCRP, causing CBS to be exposed completely, promoting the binding of mCRP with lipid rafts and the execution of its proinflammatory activity via raft–associated signaling pathways. However, the proinflammatory activities of reduced mCRP might be limited by proteolysis or aiding opsonization. Therefore, this model not only describes the pCRP dissociation process that underlies many pathophysiological conditions and the generation of mCRP in vivo, but also explains how proinflammatory activity can be precisely regulated by sequential conformational changes in CRP. Through this mechanism, the proinflammatory activity of CRP is localized to inflammatory foci, avoiding systemic inflammatory effects caused by fluctuations in CRP concentrations.

Perspective

At present, the mechanism of how CRP participates in proinflammatory responses has been studied in detail. It is concluded that pCRP is a basic structural form, whilst mCRP represents the active structural form. The change in conformation from pCRP to mCRP is an important regulator of the proinflammatory activity of CRP and explains why elevated CRP in the plasma does not lead to systemic inflammatory responses. However, the systematic overexpression of CRP appears wasteful if conformation changes are the only mechanism by which CRP participates in inflammatory responses. The in vivo data published recently demonstrated that sieving effect of basement membrane beneath endothelial cells retards the transcytosis of serum CRP towards underlying tissues [44]. This indicate that CRP deposited in inflammation lesions is mainly produced in situ, rather than from the transcytosis of circulating CRP. In addition to liver, in fact, CRP also be expressed in other tissue or cells including kidney [45], lung [46], neuronal [47], adipocytes [48], and leukocytes [49]. Therefore, sieving effect of basement membrane indicates the functional differences between CRP originated from liver and from extra-hepatic cells, and highlights the potential importance of locally produced CRP in inflammation. However, we now know little about the difference of CRP from different sources.

Conservative analysis demonstrated that cysteine (Cys36, Cys97), CBS (aa 35–47), Ca^{2+} binding sites, PC binding sites and C1q binding sites are highly conserved in CRP from zebrafish and humans [50, 51]. However, in zebrafish, multiple genes (*crp* 1–7) encoding CRP-like proteins, and their transcript variants have been detected [51]. Highly variable phospholipid binding sites exist between different CRP-like proteins indicating different phospholipid binding specificities. This is important for zebrafish with less advanced adaptive immune systems and incomplete antibody. It is, therefore, speculated that CRP-like proteins may represent the original model of the adaptive immune response and are ancestors of antibody. Accordingly, CRP may be a link between innate and adaptive immunity. Zhang et al. [52] reported that pCRP can bind to Jurkat cells and naïve T cells independent of Ca^{2+} and PC, resulting in their differentiation to Th_2. This reflects the contribution of CRP in shaping the adaptive immune response. However, further details regarding the involvement of CRP in modulating adaptive immunity are now required.

Current evidence has shown that pCRP can be dissociated to mCRP at inflammatory foci, which contributes to the exposure of its proinflammatory activities. The tight correlation of mCRP and inflammation indicate that mCRP is a more specific marker of underlying inflammatory pathological processes than pCRP. Human serum mCRP has been successfully quantified by ELISA based on commercially available antibodies revealing it to be a reliable marker of skin-related autoimmune disorders [18]. This indicated that mCRP may represent a new biomarker for the diagnosis and prognosis of inflammatory–related diseases.

As CRP is a direct participant in inflammation, the development of specific drugs targeting CRP dissociation represents a strategy of disease treatment [43]. Recent studies demonstrated that synthetic cholesterol binding sequences can inhibit mCRP–induced adhesion of endothelial cells in vitro and the release of IL-6 in mice by abrogating mCRP binding to lipid raft [35]. In addition, the peptide 201–206 can inhibit the adhesion of neutrophils to endothelial cells and platelets by interacting with FcγRII (CD32) [41]. Furthermore, 1,6 bis-phosphocholine (bisPC), a specific small molecule CRP inhibitor that crosslink two pentameric CRP by binding to phosphocholine binding site, can prevent the dissociation of pCRP and abrogate the increase of

myocardial injury induced by CRP [24, 53]. The positive effect of bisPC may be attributed to blocking of interaction between pCRP and LPC-enriched microvesicles, thus inhibiting complement-mediated tissue injury and CRP-mediated leukocyte recruitment [11, 24]. Even though some therapeutic effects were demonstrated in animal models, low half-life and low CRP affinity may restrict its clinical application [11]. However, some new designed small molecule inhibitors that inhibit pCRP dissociation or PLA_2 exposure may also have good prospects [43]. Therefore, the development of anti-inflammatory drugs targeting CRP dissociation may be a promising option for future therapies.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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